Genome-wide SNP-Based Linkage Scan Identifies a Locus on 8q24 for an Age-Related Hearing Impairment Trait

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Age-related hearing impairment (ARHI), or presbycusis, is a very common multifactorial disorder. Despite the knowledge that genetics play an important role in the etiology of human ARHI as revealed by heritability studies, to date, its precise genetic determinants remain elusive. Here we report the results of a cross-sectional family-based genetic study employing audiometric data. By using principal component analysis, we were able to reduce the dimensionality of this multivariate phenotype while capturing most of the variation and retaining biologically important features of the audiograms. We conducted a genome-wide association as well as a linkage scan with high-density SNP microarrays. Because of the presence of genetic population substructure, association testing was stratified after which evidence was combined by meta-analysis. No association signals reaching genome-wide significance were detected. Linkage analysis identified a linkage peak on 8q24.13-q24.22 for a trait correlated to audiogram shape. The signal reached genome-wide significance, as assessed by simulations. This finding represents the first locus for an ARHI trait.

Age-related hearing impairment (ARHI), or presbycusis, is the progressive bilaterally symmetric deterioration of hearing ability that occurs with aging. Both studies of the cochlea in many animal species and histopathological studies of human temporal bones have shown that stria vascularis volumes, spiral ganglion cell, inner hair cell and outer hair cell populations, as well as many other cochlear cell types and structures show age-related degeneration.[1–3]

Environmental risk factors for human ARHI have been studied extensively. Among these are noise exposure, smoking, ototoxic medication, and cardiovascular disease and its risk factors.[1,4,5] Only very recently investigators have started to elucidate the genetic architecture of human ARHI. Familial aggregation of ARHI is well established now. Heritability estimates vary between 0.25 and 0.75, depending, among other factors, on study design (families versus twins), age range of the study population, and the phenotype studied.[6–9]

To date, two linkage studies for ARHI have been published.[10,11] These studies resulted in a number of weakly suggestive linkage peaks. A number of candidate gene-based association studies has been carried out, which are reviewed in Van Eyken et al.[4] Recently, by using a candidate gene approach, we reported associations between hearing ability and variants in the *GRHL2* (MIM 608576) gene.[12]

In this paper, we present the results of a cross-sectional family-based genetic study employing audiometric data. With high-density SNP data, a genome-wide linkage as well as an association scan were performed. Principal component analysis was used to reduce the dimensionality of the multivariate phenotype, while capturing most of the information contained in the audiometric data. This study reports a genome-wide significant linkage to an ARHI trait.

DNA samples were collected from 1126 subjects of European descent from 204 large sibships by 9 centers in 7 European countries. Neither families nor subjects were ascertained based on phenotype. Samples were collected via population registries or via audiological consultations. The study was approved by the ethics committees or the appropriate local institutional review boards at all participating universities or hospitals. Informed consent was obtained from all study participants. All subjects underwent an otoscopic investigation and completed an extended questionnaire detailing medical history and exposure to

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environmental factors. Subjects with medical conditions or pathologies that could potentially affect hearing ability were excluded. Air-conduction pure-tone hearing thresholds were measured at 0.25, 0.5, 1, 2, 4, and 8 kilohertz (kHz). Bone conduction was tested at 0.5, 1, 2, and 4 kHz. Audiological exclusion criteria were an air-bone gap of more than 15 dB (dB) averaged over 0.5, 1, and 2 kHz in one or both ears or asymmetrical hearing loss with a difference in air-conduction thresholds exceeding 20 dB in at least 2 frequencies between 0.5, 1, and 2 kHz. After genotypic data cleaning, phenotypic data (air-conduction pure-tone hearing thresholds) were available for 955 subjects. This sample contained 430 men (45%) and 525 women (55%) with a median age of 61 (ranging from 49 to 76). The median number of phenotyped sibs per sibship was 5 with a maximum of 10. All subjects were genotyped with the Affymetrix 250K *Sty* chip (238,000 SNPs). To aid in determining identity-by-descent status, these subjects included 126 unphenotyped sibs and parents. Genotype calling was done with the BRLMM algorithm of Affymetrix.

Motivated by the assumption that unilateral hearing loss primarily reflects environmental effects, for each subject, hearing thresholds for the better-hearing ear were selected for the analysis, based on the average over all frequencies. Individual hearing thresholds Y were transformed as follows: $\log_{10}(20 + Y)$. Mean collecting center, age, age squared, and age cubed effects were regressed out. This was carried out for males and females separately. To deal with the significant heteroscedasticity that was present mainly in the high frequencies, residuals were scaled. Subsequently, classical principal component analysis was conducted on the combined centered and scaled residuals. In addition, the principal components of heritability as proposed by Ott and Rabinowitz[13] were calculated. These analyses were carried out with R and SAS version 9.1.3. The calculation of the principal components of heritability entails solving a generalized eigenproblem that was solved numerically with the GENEIG subroutine of SAS. Variance components model-based heritability calculations were carried out with the MIXED procedure in SAS and heritability was estimated as twice the intraclass correlation.[14]

Quality control was carried out with the PLINK toolset.[15] Six subjects were excluded because of poor sample call rate (<94%). The average genotyping rate of the remaining samples was 99.13%. Nonpositioned SNPs (74) and SNPs with more than 10% missingness (1740) were removed from the analysis. Familial relationships were checked with GRR[16] with 3000 randomly selected SNPs. Fourteen subjects were removed because of relationship inconsistencies. There were four pairs of monozygous twins in the data. For each of these pairs, the subject with the lowest sample call rate was removed from the analysis. Concordance rates for the four pairs varied from 99.71% to 99.81%. One subject was removed from the analysis because it had a large negative inbreeding coefficient, which is indicative of sample contamination. Five subjects were excluded because they no longer had relatives in the sample set. A further four subjects were removed because no sex was specified. A sex check identified six discrepancies. For these subjects, sex was imputed based on the SNP data.

Multidimensional scaling on a genetic distance matrix in which each entry corresponded to 1 minus the genome-wide mean proportion of alleles shared identical-by-state (IBS) revealed a family with possible non-European ancestry that was excluded from further analyses (Figure S1 available online). These steps left 1081 genotyped subjects from 200 sibships amenable to analysis.

Multidimensional scaling revealed a clear separation between samples from Western European countries (WEU) and from Finland (FIN) and a south-north axis within WEU (Figure S1). Therefore, it was decided to conduct Hardy-Weinberg equilibrium (HWE) testing for samples from WEU and from FIN separately. One individual per sibship was randomly selected and an exact test[17] was used on this subsample of unrelated individuals. This process was repeated 10 times and the geometric mean of the resulting 10 p values was regarded as the final p value for HWE tests for each SNP.

SNPs with minor allele frequencies (MAF) $> 5\%$ and HWE p values > 0.001 in both WEU and FIN were used in the association analysis (169,154 SNPs). For linkage analysis, a subset of informative SNPs was used. Based on the Marshfield genetic map, chromosomes were divided into segments 0.1 cM in length and for each segment the first SNP with MAF $> 15\%$ and HWE p value > 0.05 in both WEU and FIN was selected. This yielded a map of 26,879 SNPs with an average marker spacing of ~0.14 cM. This map density should be sufficient to extract most of the inheritance information[18] and approaches the upper limit of what current linkage analysis software can handle. To assess the impact of the choice of SNPs on the final results, analyses were repeated with two other map densities. A second set of 15,839 SNPs with an average marker spacing of ~0.24 cM was analyzed (4250 SNPs overlapped with the previous set), as well as a third set for which every other marker of the second set was selected yielding a map of 7,723 SNPs with an average spacing of ~0.49 cM (autosomes only). The results for the three different maps were virtually identical (see Figure S2 for PC3). The multipoint entropy-based measure of marker informativeness reported for each analysis position by MERLIN[19] was high throughout the genome, generally being well above 90% (Figure S2).

Possible genotyping errors were detected by looking for unlikely double recombinants via MERLIN. During this process, linkage disequilibrium (LD) was modeled via the approach of Abecasis and Wigginton[20] that groups markers in LD into clusters. As recommended, markers with $r^2 > 0.1$ were grouped into clusters. The percentage of genotypes identified as erroneous was 0.29%. These genotypes were set to missing.

Quantitative trait linkage analysis was carried out with the regression-based procedure of Sham et al.[21] that has been implemented in MERLIN-REGRESS.[21] Because the

presence of LD may result in upward biases in multipoint linkage analysis, the analysis was carried out both ignoring any LD between markers and via the approach of Abecasis and Wigginton where SNPs with $r^2 > 0.1$ were organized into clusters.[20] Results obtained with both approaches were virtually identical (Figure S3). To deal with potential outliers, quantile normalization was applied to trait values. The X chromosome was analyzed separately with a variance-components model in MINX, the X-specific version of MERLIN.

To determine the empirical genome-wide significance level, we conducted simulations. With MERLIN, we generated 1000 simulated data sets under the null hypothesis. In each simulation run, data were generated for WEU and FIN separately. The data for WEU and FIN were subsequently combined and analyzed via MERLIN-REGRESS. The empirical false positive rate for a LOD score L was calculated with the formula $(r + 1)/1001$, where r is the number of times a simulation run had one or more LOD scores greater than L.[22,23] Because this task was very computer intensive, we used the sparsest map and ignored LD.

Quantitative trait association testing was performed with MERLIN, which implements a variance component model that models background polygenic effects.[24] To deal with potential outliers, quantile normalization was used throughout. The X chromosome was analyzed separately with MINX. Throughout all calculations, an additive model was assumed. To avoid spurious associations resulting from population stratification, association was tested for WEU (751 subjects) and FIN (204 subjects) samples separately. Results were subsequently combined by meta-analysis. To assess the presence of residual population stratification within the WEU and FIN samples, the resulting empirical p value distributions were inspected by quantile-quantile plots and by calculating genomic control inflation factors.[25] To combine evidence for association in WEU and FIN, we used the meta-analytic approach described in Sanna et al.[26] In agreement with other studies, we took a p value cutoff of 5×10^{-7} to declare genome-wide significance.[27] We further explored the data by carrying out an analysis stratified by gender. The annotation software tool WGAViewer[28] was used to aid in interpreting the association results.

Hearing thresholds were corrected for age, gender, and collection center, and classical principal component analysis (PCA) was performed on the scaled residuals. The resulting eigenvalues, the loadings (coefficients of the eigenvectors), and heritability estimates for the first three classical principal components (PCs) are given in Table 1. The eigenvalue for a PC corresponds to the proportion of the total variability explained by that PC. The first three PCs capture 87.7% of the variation. PC1 is clearly a "size" variable, an overall measure of one's hearing ability. PC2 and PC3 are audiogram shape variables: PC2 contrasts low with high frequencies, whereas PC3 contrasts the middle with the low and the high frequencies. Figure 1 shows how these traits relate to the audiograms. Based on non-

Table 1. Eigenvalues, Coefficients of the Eigenvectors, and Heritabilities of the First Three Classical Principal Components and of the First Three Principal Components that Maximize the Heritability

Trait	PC1	PC2	PC3	PCH1	PCH2	PCH3
Eigenvalue	0.603	0.174	0.100	0.524	0.241	0.155
Coefficients Eigenvector						
0.25 kHz	−0.397	0.397	0.551	0.590	0.915	0.497
0.5 kHz	−0.448	0.384	0.126	0.198	0.193	−1.267
1 kHz	−0.451	0.241	−0.354	0.238	−0.406	0.096
2 kHz	−0.440	−0.123	−0.531	0.381	−0.845	0.251
4 kHz	−0.378	−0.510	−0.118	0.108	−0.241	0.142
8 kHz	−0.319	−0.602	0.510	−0.046	0.451	0.779
Heritability	0.663	0.272	0.375	0.703	0.391	0.269

standard likelihood ratio tests, there was significant familial aggregation for all traits (all p values < 0.001). Heritability was highest for PC1 (66.3%) and lowest for PC2 (27.2%).

Principal components of heritability (PCHs) were calculated as well because these may offer a significant power advantage if the individual variables comprised in the multivariate phenotype vector are correlated.[13] The results for the first three PCHs are given in Table 1. Compared to the classical PCs, the PCHs have a less clear-cut biological interpretation. Table 2 shows the correlations between the PCs and the PCHs. It can be seen that PC1 is strongly correlated with PCH1 (Spearman correlation of 0.95), PC2 is moderately correlated with PCH3 (0.79), and PC3 is strongly correlated with PCH2 (0.95). This was also reflected in the linkage results: LOD score curves were virtually identical between correlated PCs (not shown). We therefore pursued only the classical PCs because these have a more straightforward interpretation. In addition, they allow us to compare results with future association studies based on unrelated subjects, because PCHs cannot be calculated for unrelated subjects.

Quantitative trait linkage analysis was carried out for the first three PCs via the reverse Haseman-Elston method of Sham et al.[21] The only trait for which multipoint LOD scores greater than 3 were observed was PC3 (Figure S4; Figure S3 shows the results for PC1 and PC2). Based on the sparsest map used, on chromosome 8 a multipoint LOD score of 4.23 was attained at the position of SNP rs4512366 (physical position 125528900; pointwise p value $= 5\times10^{-6}$). On chromosome 9, a multipoint LOD score of 3.13 was attained at the position of SNP rs10814227 (physical position 35320608; pointwise p value $= 7\times10^{-5}$).

We conducted 1000 simulations to assess the empirical genome-wide significance level of these linkage peaks. For the data at hand, in order to reach genome-wide significance at the 5% level, a LOD score should exceed 3.55 (see Figure S5). The maximal LOD score for chromosome 8 reached genome-wide significance (p value = 0.0170). The peak on chromosome 9 did not (p value = 0.1039).

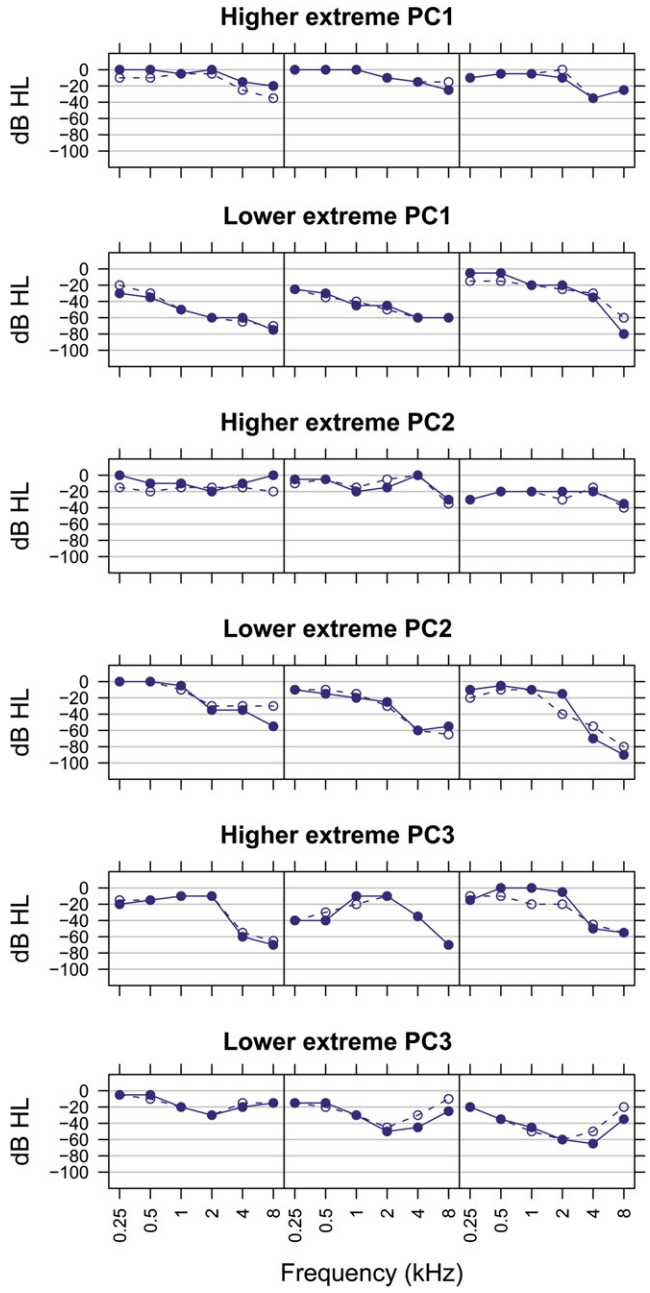

Figure 1. Comparison of Audiograms between Subjects with High and Low Values for PC1, PC2, and PC3

Three subjects were randomly selected from the lower and upper 10% extremes of the distribution for each of the three traits. To show the extent of intrasubject variability, audiograms for both ears are given. Left and right ears correspond to filled and open circles, respectively. For PC1, on average, hearing loss is much less pronounced in individuals from higher extremes (top row) compared to the lower extremes (second row from the top). Subjects with low values for PC2 (fourth row from the top) tend to have a much more sloping audiogram, compared to subjects with high values for PC2 (third row from the top). Subjects with high values for PC3 (second row from the bottom) tend to have a more concave audiogram, compared to subjects in the lower extreme for PC3 (bottom row).

Table 2. Spearman Rank-Order Correlations between the First Three Classical Principal Components and the Principal Components of Heritability

	PCH1	PCH2	PCH3
PC1	−0.953	0.113	−0.171
PC2	0.203	0.264	−0.790
PC3	−0.013	0.947	0.219

The region reaching significance on chromosome 8 spanned approximately 7 Mb, roughly between SNPs rs3765212 and rs4601326. This corresponds to cytogenetic chromosome bands 8q24.13-q24.22 (UCSC Genome Browser).

We further explored the linkage peak for PC3 on chromosome 8. Figure 2 shows multipoint and singlepoint LOD scores together with informativities for chromosome 8. The singlepoint analysis revealed that three SNPs have high singlepoint LOD scores of 5.26, 5.24, and 4.75, respectively. We repeated the analysis, leaving out these three SNPs. The results of the multipoint analysis were barely affected (maximal multipoint LOD score of 4.22). Inspecting SNP informativities is not very enlightening here and informativity of individual SNPs is low. A likely explanation for the three high singlepoint LOD scores is that the informativity of the corresponding SNPs in the (unknown) subset of pedigrees in which a locus on 8q is truly linked is high compared to surrounding markers. Table S1 provides additional information on each family and family-specific contributions to the multipoint LOD score at the positions of the SNPs beneath the linkage peak. The latter are based on a variance components analysis that yielded results that were strikingly similar to the regression-based analysis (see Figure S6).

Quantitative trait association testing was performed with a variance component model to account for background polygenic effects. Genomic control inflation factors[25] varied from 1.011 to 1.035, suggesting that residual population stratification is of minor concern. No association signals meeting genome-wide significance were observed for any of the three traits, neither in the analyses stratified by origin and gender, nor in the meta-analysis (see Figure S7 for plots of meta-analysis $-\log_{10}$(p values) for the three traits). Figure S4 shows for PC3 the results of the linkage analysis superimposed on those of the association analysis, together with the positions of the 45 known nonsyndromic hearing-loss genes. We further scrutinized the region beneath the significant as well as the suggestive linkage peak for clustering of semi-independent association signals with p values $< 10^{-3}$ in a gene region, taking into account LD, and for hits in or near plausible candidate genes. We found no evidence for association with PC3 in this region.

We found a genome-wide significant linkage signal on 8q24.13-q24.22 for PC3, a trait related to audiogram shape that was obtained via principal component analysis. These

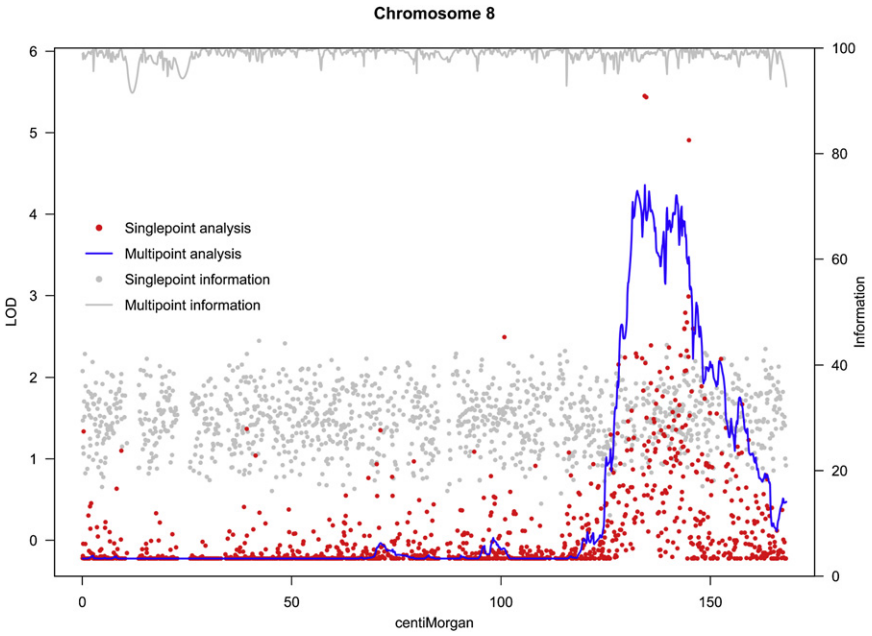

Figure 2. Chromosome 8 Multipoint and Singlepoint LOD Scores for PC3 together with Marker Informativities
Gray and red dots represent singlepoint marker informativities and LOD scores, respectively. Gray and blue solid lines represent multipoint informativities and LOD scores, respectively.

results suggest that one or several rare variants that have a moderate to large effect on audiogram shape in the elderly reside in this broad chromosomal region.

To date, two linkage studies for ARHI have been published. Garringer et al.[11] reported suggestive linkage (LOD score = 2.5) in the 3q22 region based on a sample of male dizygotic twins concordant for self-reported hearing loss. The study of DeStefano et al.[10] used extended pedigrees from the Framingham cohort. Based upon the average pure-tone hearing thresholds for the middle and low frequencies (after correction for age and gender), several regions of suggestive linkage were found, the highest of which reached a LOD score of 2.1 on 11q13. Because the phenotypes analyzed in the two linkage studies cited above correlate with PC1 (unpublished results), suggestive linkage peaks from these studies were compared with our results. We did not find any evidence in favor of true linkage signals.

Whereas linkage analysis is the preferred strategy for identifying rare genetic variants with large effects, genome-wide association (GWA) analysis is the method of choice for identifying common genetic variants with only small effects. The absence of any genome-wide significant association signals in this study may be due to a variety of factors, which may act in concert. The Affymetrix 250K *Sty* chip has low genomic coverage and this presents the major limitation of our association study. The modest sample size presents a further limitation. In the light of what we now know about typical effect sizes for complex diseases and traits, power to detect any causative variants in this association study is probably very low.[29–31] Furthermore, because it is plausible that the culprit variants for ARHI are not manifesting themselves during reproductive age, the resulting absence of any selective pressure could have easily led to the propagation of many harmful variants in human populations by genetic drift or genetic hitchhiking. Because the samples studied have different ethnicities, substantial genetic and allelic heterogeneity may wash out any effect. Synergistic gene-gene and gene-environment interactions may further dilute the effect and reduce power. Last, but not least, the precision of hearing threshold measurements is low (5 dB). Therefore, despite the substantial role of genetics in the etiology of ARHI as indicated by heritability studies, the signal-to-noise ratio may be too low to pick up any signal of the individual contributing variants.

Based on a candidate gene approach, we previously reported associations between variants in the *GRHL2* gene, a known monogenic nonsyndromic hearing loss gene, and hearing ability.[12] In this study, subjects were assigned case or control status based on whether they belonged to the upper or lower extreme of the distribution of the average of the high-frequency hearing thresholds, corrected for age and gender. Because this variable is correlated to PC1 (unpublished results), we looked for evidence of replication. The top hit from the candidate gene study (rs10955255) was not among the SNPs analyzed in the present study. We scrutinized association signals in the region of this SNP but failed to find any evidence in favor of replication. However, the maximum r^2 between the currently tested SNPs and rs10955255 was only 0.44.

The present study differs from previous genetic studies for ARHI in an important way. Previous studies were based on either self-reported hearing loss or simple averages of age- and gender-corrected hearing thresholds for different frequencies. By using such strategies, many biologically relevant features of the audiometric data will be lost because completely different audiometric shapes may lead to similar phenotypic values when thresholds are averaged over frequencies. By using principal component analysis, we obtained two measures for audiogram shape, PC2 and PC3, which measure slope and concavity, respectively. A number of studies in which human temporal bones were compared to premortem hearing tests have shown that audiometric patterns correlate with pathophysiology.[2,32] Nelson et al.[2] studied individuals with downward sloping audiometric patterns and reported that the severity of hearing loss, based on audiometric thresholds, was highly

associated with the degeneration of stria vascularis volumes and outer and inner hair cell and ganglion cell populations. The slope of the audiogram was associated with the extent of ganglion cell degeneration. Therefore, it is plausible that PC2 and PC3 contain critically important clues to elucidate the molecular etiology of ARHI.

The significant heritability estimates for the three traits studied suggest that the role of genetics in the etiology of ARHI is substantial. In view of the fact that a plethora of genes plays a role in the hearing system, ARHI probably will turn out to be a very heterogeneous disorder. For nonsyndromic monogenic hearing loss only, 45 genes have been identified (Hereditary Hearing Loss webpage). Because of the great difficulty of separating the age effect from harmful environmental effects, a big challenge remains the collection of high-quality data. In this respect, the collection of cohort data should be heavily encouraged because the study of individual longitudinal profiles may yield important insights that could lead to the discovery of different genetic subtypes of ARHI.

Supplemental Data

Supplemental Data include seven figures and one table and are available at http://www.ajhg.org/.

Acknowledgments

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Web Resources

The URLs for data presented herein are as follows:

GRR, http://www.sph.umich.edu/csg/abecasis/GRR/
Hereditary Hearing Loss webpage, http://webh01.ua.ac.be/hhh/
MERLIN, http://www.sph.umich.edu/csg/abecasis/Merlin/index.html (for MERLIN-REGRESS, MERLIN, and MINX)
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/
PLINK, http://pngu.mgh.harvard.edu/purcell/plink/
R, http://www.R-project.org
UCSC Genome Browser, http://genome.ucsc.edu/cgi-bin/hgGateway

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